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# BRIEF REPORT



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A stretch of purine residues, the polypurine tract (PPT), is found in all retroviruses and is used to initiate plus-strand DNA synthesis. While the PPT of most lentiviruses is a homogeneous sequence of purine residues, the PPT of some isolates of the human and simian immunodeficiency viruses is interrupted with a single pyrimidine residue. The ROD strain of human immunodeficiency virus type 2 (HIV-2) has such a pyrimidine-containing variant PPT. Virus generated from an infectious molecular clone, pROD10, was used to infect two CD4-positive T-cell lines, H9 and CEM. The sequence of the PPT was determined after two passages. From both cell lines, the variant PPT was retained, demonstrating that the presence of a pyrimidine in the PPT was fully functional and that there was no strong selection for an all-purine PPT. 

The infectious cycle of retroviruses involves a complicated series of events that begins with the interaction of the virus with its cellular receptor(s) and results in the production of infectious virions (for reviews see Coffin, 1996; Luciw, 1996; Weiss *et al.,* 1984). Following fusion of the retrovirus envelope with the cellular membrane, the nucleocapsid core is released into the cytoplasm. The single-stranded RNA genome, which is flanked by short terminal repeats (termed R), is reverse transcribed into double-stranded linear DNA, in the process duplicating elements at each end of the viral RNA to form the long terminal repeat (LTR) elements, which are found at each end of the viral DNA (Fig. 1). It is this two-LTR linear molecular that is the precursor to integration into the host cell genome (for reviews see Coffin, 1996; Luciw, 1996; Whitcomb and Hughes, 1992). As with other DNA polymerases, the reverse transcriptase (RT) of human immunodeficiency virus (HIV) requires a primer to initiate DNA synthesis, and as with other retroviruses, HIV uses a host cell tRNA to prime minus-strand DNA synthesis. In the case of the lentiviruses, including HIV-1 and HIV-2, this is usually $tRNA^{Lys3}$ (Myers *et al.,* 1995), although other tRNA species can be used *in vivo* (Lauermann *et al.,* 1996) or *in vitro* (Wakefield *et al.,* 1996). The 3′ 18 nucleotides of the tRNA anneal to a complementary sequence in the genomic RNA just downstream of U5 in a region called the primer binding site (PBS; for reviews see (Coffin, 1996; Luciw, 1996; Weiss *et al.,* 1984; Whitcomb and Hughes, 1992). This primer is then extended to the end of the RNA template. Following the first (minus-strand) transfer and elongation, the primer for the plus-strand DNA is generated by specific RNaseH cleavage of the genomic RNA, which is now part of a duplex with the minus-strand DNA. The primer derives from a purine-rich sequence, the polypurine tract (PPT). The PPT is located just upstream of U3 in the RNA genome (and of the 3′ LTR in the proviral genome) and is required for the replication of retroviruses (Sorge and Hughes, 1982) and retrotransposons (Lauermann *et al.,* 1995). The correct position of the cleavage downstream of the PPT is critical, since it determines, together with the removal of the primer, the sequence of the inverted repeat at the left end of the viral DNA. This repeat is required in *cis* by the viral integrase protein to insert the viral genome into the host genome (Roth *et al.,* 1989; Varmus and Brown, 1989). HIV and some other retroviruses have a second polypurine tract, PPT2, located near the center of the genome (Guyader *et al.,* 1987; Ratner *et al.,* 1985; Sanchez-Pescador *et al.,* 1985; Wain-Hobson *et al.,* 1985) that enhances viral replication (Charneau *et al.,* 1992, 1994; Hungnes *et al.,* 1992). For clarity, we refer to the PPT upstream of U3 as PPT1 (Fig. 1).

Several biochemical studies (Powell and Levin, 1996; Rattray and Champoux, 1987, 1989) have been conducted to measure the precision of RNaseH cleavage and the subsequent extension from mutated polypurine tracts using the MoMLV and HIV-1 PPT elements with purified RT's. The results of these experiments suggest that, even though some mutations in the PPT are tolerated, in many cases the efficiency of cleavage is reduced

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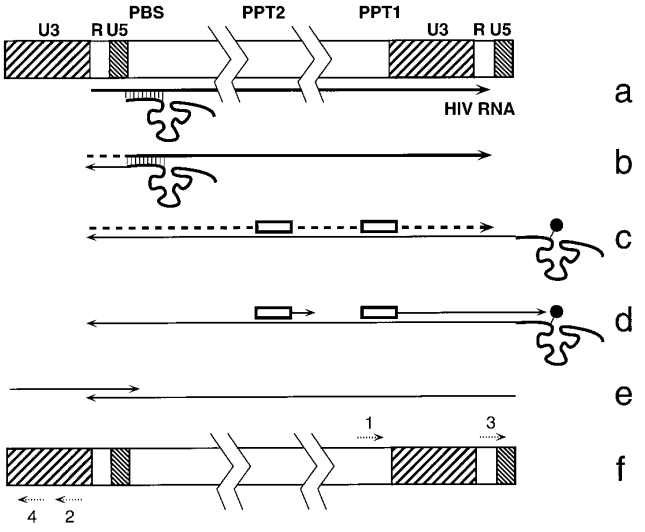


FIG. 1. Model of retroviral reverse transcription. (a) The DNA structure of the HIV provirus with its long terminal repeat (LTR; consisting of U3, R, and U5) elements. The cellular tRNA molecule that acts as the initial primer for minus-strand DNA synthesis is annealed to the primer binding site (PBS) of viral genomic RNA (thick line). (b) Minus-strand strong-stop DNA (thin line) is created when synthesis reaches the end of RNA template; the RNA in a duplex with DNA is degraded (dashed line) by the virus-encoded RNaseH. (c) Minus-strand strong-stop DNA is transferred to the 3′ end of the genomic RNA and is extended along this template. The open rectangles represent the polypurine tracts (PPT1 and PPT2), which are RNaseH-resistant RNA elements and become the plus-strand primers. (d) Plus-strand synthesis commences from the polypurine tracts. PPT1 is found just upstream of the 3′ LTR, and PPT2 is located approximately in the middle of the genome. (e) After the second (plus-strand) transfer, full-length viral DNA is generated (f). Positions of the primers used for polymerase chain reaction (PCR) amplification and sequencing of the two-LTR circular DNA (see Fig. 2 legend for details) are designated by dotted arrows. The primers 1 and 2 were used for the first round of PCR; the primers 3 and 4 was used for the second PCR amplification and for sequencing.

and the specificity relaxed. Purine residues close to the 3′ end of the PPT appear to be critical, whereas those farther from the point of priming can be exchanged with pyrimidines (Palaniappan *et al.,* 1996; Powell and Levin, 1996; Pullen *et al.,* 1993; Rattray and Champoux, 1989). The activity of the mutant PPT1 elements was not assessed with respect to virus replication. In this study, we addressed the question of whether a virus with a noncanonical, pyrimidine-interrupted PPT1 is able to replicate and whether such a PPT is retained upon passage of the virus or if selection pressure would cause it to revert to the canonical sequence. In addition, we have determined the specificity of cleavage at the PPT1 by RNaseH by sequencing the ends of the LTR elements in unintegrated viral DNA.

HIV-$2_{ROD}$ was isolated from a west African male with AIDS (Clavel *et al.,* 1986a). Three independent clones have been isolated by different groups from CEM cells acutely infected with HIV-$2_{ROD}$ (Clavel *et al.,* 1986b; Daniel *et al.,* 1988; Naidu *et al.,* 1988; Ryan-Graham and Peden, 1995). The reported sequence of the ROD strain has a noncanonical PPT1. This sequence differs from the HIV-2 consensus PPT1 in the arrangement of the purine residues (Myers *et al.,* 1995), as well as being interrupted by a single pyrimidine (Guyader *et al.,* 1987) (Fig. 2A). We determined the sequence of the PPT1 of two infectious molecular clones of HIV-$2_{ROD}$, pROD2, and pROD10 (Daniel *et al.,* 1988; Naidu *et al.,* 1988; Ryan-Graham and Peden, 1995). While this sequence was the same in these two clones (Fig. 2A), it differed from the published HIV-$2_{ROD}$ PPT in the number of A and G residues (Guyader *et al.,* 1987). The difference with the original sequence is not understood but may be due to the difficulty in reading runs of purines in sequencing gels.

Because three independent clones isolated from cells infected with HIV-$2_{ROD}$ possessed a pyrimidine-interrupted PPT1, it is likely that the predominant viruses circulating in the patient had this variant PPT1. To assess the stability of the PPT1 *in vitro,* virus was prepared by transfection of 293 cells with the plasmid pROD10 and used to infect the CEM and H9 T-cell lines at an approximate multiplicity of infection (m.o.i.) of 0.01 (Peden and Martin, 1995). Virus production was followed by the presence of RT activity in the medium. Virus was collected at Day 10 after infection, which was at the time of peak RT activity in each of the cultures. The virus stocks from their respective cultures were used to infect fresh CEM and H9 cells at an m.o.i. of about 0.01. At the peak of virus production, the medium was collected, and DNA was prepared (Whetsell *et al.,* 1992) from the cells. We estimate from the growth curves (not shown) that these two passages corresponded to between 10 and 20 virus generations (Dimitrov *et al.,* 1993). The DNA was subjected to PCR to amplify the PPT1 region followed by PCR sequencing using the automated ABI fluorescent dideoxy method (Lauermann and Boeke, 1994; Wei *et al.,* 1995).

The results were unambiguous: the PPT1 was maintained after the second passage in both CEM and H9 cells, and there was no evidence that the pyrimidine residue mutated to a purine (Fig. 2C), demonstrating that a PPT1 containing a pyrimidine was functional for virus replication.

Several factors need to be addressed if there is broad biological relevance to our finding. The issue of whether the HIV-$2_{ROD}$ noncanonical pyrimidine-interrupted PPT1 exists *in vivo* or is a result of *in vitro* passage in T-cell lines cannot be determined, as patient material is unattainable. The following points taken collectively suggest that it is likely that this PPT1 sequence was present in the original virus. (1) The original isolate was obtained by coculture of the patient's peripheral blood mononuclear cells (PBMC) with uninfected activated PBMC (Clavel *et al.,* 1986a). Subsequently, this virus was found to infect T-cell lines and infected CEM cells were the source of the virus for the cloning of the viral genome (Clavel *et al.,* 1986b; Naidu *et al.,* 1988). (2) Three inde-

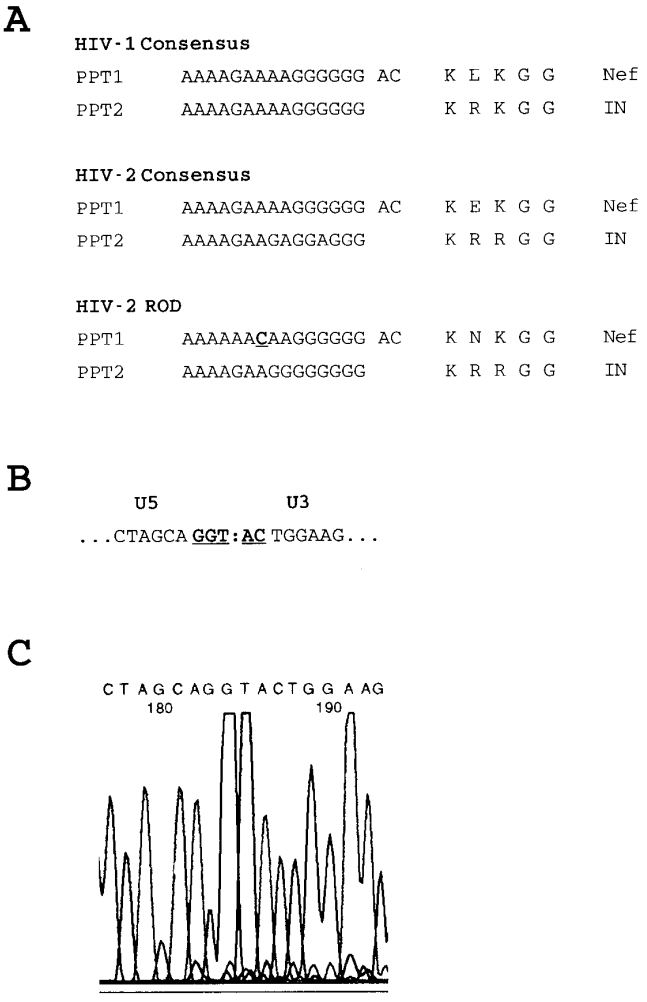


FIG. 2. Nucleotide sequences of the polypurine tracts of HIV-1 and HIV-2 and the two LTR circle junction of HIV-$2_{ROD}$. (A) The sequences of the polypurine tracts of HIV-1 and HIV-2. The consensus sequences of the PPT1 and PPT2 of HIV-1 and HIV-2 (Myers *et al.,* 1995) as well as the sequences of the PPT1 and PPT2 of HIV-$2_{ROD}$ are shown. The gap in the sequence of the PPT1 corresponds to the RNaseH cleavage site. The corresponding amino acid sequence of the overlapping genes (*nef* for PPT1 and integrase for PPT2) are also shown. As explained in the text, the sequence of HIV-$2_{ROD}$ is different from the one in GeneBank (Accession No. M15390). (B) The circle junction sequence of HIV-$2_{ROD}$. DNA was prepared from infected cells, and the region containing the junctions was amplified by two rounds of PCR using oligonucleotides VL7 5′-GGCAGG-CCTGACTTGTGCCC-3′ and VL8 5′-GCACTTGGCCGGTGCTGGGC-3′ followed by amplification with VL11 5′-GACGGCCCCACGCTTGCTTGC-3′ and JB1000 5′-TCTAAGTATATATTTAAGATTTTATGTCT-3′. The products were sequenced directly using IBI sequencing system. The sequence of part of the U5 and U3 regions is shown. The five nucleotides not found at the ends of the proviral DNA are underlined, and the ends of the DNA are indicated with a colon. Incorrect cleavages were estimated to be no more than 10%. (C) The region of the sequencing gel that shows the circle junction. The background peaks seen on the right side of the chromatogram are most likely not due to heterogeneity of the sequence, since similar background differences were seen elsewhere in the chromatogram (not shown).

pendent clones of HIV-$2_{ROD}$ were all found to have the noncanonical pyrimidine-interrupted PPT1. (3) Similar noncanonical pyrimidine-interrupted PPT1 elements (with pyrimidines at different positions) have been found in a few other molecular clones of HIV-2 and SIV; however, the stability of the PPT1 in those cases has not been addressed.

It could be argued that not enough replication cycles have been performed to allow reversion of the PPT1. However, changes were found in the *gag* gene from these passaged viruses (unpublished results), rendering this contention unlikely.

Since the *nef* gene overlaps the PPT1, maintenance of the PPT1 sequence could be due to selection either for the PPT1 or for the overlapping gene. There are two infectious molecular clones with this pyrimidine-containing PPT1, one with the *nef* reading frame open, pROD10, and the other carrying *nef* deletions, pROD2. In this latter case, selection could only have involved the PPT1. Deletions in the *nef* gene have been found *in vivo* (Deacon *et al.,* 1995; Kirchhoff *et al.,* 1995) but to our knowledge do not arise on passage *in vitro.* This suggests that it is the particular combination of RNaseH and the PPT1 for HIV-$2_{ROD}$ that had been selected for *in vivo.*

Introducing pyrimidines into the PPT can relax RNaseH specificity (Palaniappan *et al.,* 1996; Powell and Levin, 1996; Pullen *et al.,* 1993; Rattray and Champoux, 1989). Therefore, it was important to determine the specificity of RNaseH cleavage for the pyrimidine-containing ROD PPT1 during retrovirus replication. During productive infection, not all of the linear viral DNA is integrated into the host chromosome; some is converted to two-LTR circular molecules, presumably by a cellular ligase. Because this ligation preserves the nucleotides normally removed from the ends of the viral DNA during integration (Hong *et al.,* 1991; Kulkosky *et al.,* 1990; Pauza, 1990; Smith *et al.,* 1990; Whitcomb and Hughes, 1991; Whitcomb *et al.,* 1990), the sequence of the circle junction provides information about the ends of the linear viral DNA. The left end of the viral genome is defined by the removal of the PPT primer by RNaseH. The DNA isolated from the infected cultures was amplified by two rounds of PCR and subjected to direct sequencing. All seven cultures in which junction sequences were analyzed had five nucleotides between the LTR elements at the circle junction that are not present at the ends of the proviral genome (Figs. 2B and 2C). These were the same five nucleotides found for the NIH-Z strain of HIV-2 (Whitcomb and Hughes, 1991), which has an all-purine and canonical PPT1 (Fig. 2A). Therefore, both types of PPT direct the same cleavages at the 3′ end of the PPT. The analysis of the sequence chromatograms of the pool of circle junctions suggests that the RNaseH cleavage is precise. Based on this type of analysis (Wei *et al.,* 1995), we estimate that incorrect cleavages can be no more than 10% of the total cleavage events. This is similar to the proportion of aberrant LTR–LTR junctions seen previously with HIV-1 (Whitcomb *et al.,* 1990) and HIV-2 (Whitcomb and Hughes, 1991).

In summary, we have shown: (1) that a noncanonical pyrimidine-interrupted PPT1 is fully functional for HIV replication *in vitro;* (2) there was no reversion to an all-purine PPT1 after two passages on two T-cell lines, CEM and H9; and (3) this noncanonical PPT1 appears to direct the same 3′ cleavage specificity as an all-purine PPT1.

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